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(54) Title: BIVALENT MOLECULES THAT FORM AN ACTIVATING COMPLEX WITH AN ERYTHROPOIETIN RECEPTOR		
(57) Abstract Bivalent molecules which activate erythropoietins and induce the proliferation or differentiation of erythroid progenitor cells are provided. Also provided are methods of using such bivalent molecules for drug discovery, diagnosis and treatment of disorders related to the activation of an erythropoietin receptor.		

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BIVALENT MOLECULES THAT FORM AN ACTIVATING COMPLEX WITH AN ERYTHROPOIETIN RECEPTOR

3

INTRODUCTION

6 Technical Field

This invention is directed to bivalent molecules that trigger cell proliferation and differentiation of erythroid precursors by activating signal transduction through the formation of a complex with erythropoietin receptors.

Background

12 Erythropoietin (EPO), a 34-kDa glycoprotein hormone, is the major regulator of mammalian erythropoiesis (Krantz, S.B. (1991) *Blood* 77, 419-434). EPO acts on erythroid progenitor cells by preventing apoptosis (Koury *et al.* (1990) *Science* 248, 378-381; Zhuang
15 *et al.* (1995) *J Biol Chem* 270, 14500-14504), stimulating proliferation of erythroid precursor cells and by inducing differentiation into mature erythrocytes. These effects are transduced by binding of EPO to a specific erythropoietin receptor (EPO-R) on the surface of committed
18 erythroid progenitor cells (Yousoufian *et al.* (1993) *Blood* 81, 2223-2236). Deletion of EPO or EPO-R genes in mice has shown that EPO is crucial for the survival, proliferation and differentiation of late committed progenitors (colony forming unit-erythroid, CFU-E), but not
21 of early progenitors (burst forming erythroid, BFU-E) (Wu *et al.* (1995) *Cell* 83, 59-67). Mice homozygous for a deletion of either EPO or EPO-R genes die during embryogenesis due to failure of erythropoiesis in the fetal liver.

24 The EPO-R is a member of the cytokine receptor type I superfamily, which includes the receptors for interleukins (IL) 2-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-stimulating factor (G-CSF), growth hormone (GH), prolactin,
27 thrombopoietin (TPO), leukemia inhibitory factor (LIF), and leptin (Bazan, J.F. (1990) *Proc. Natl. Acad. Sci.* 87, 6934-6938; Alexander *et al.* (1995) *EMBO J.* 14, 5569-5578; Tartaglia *et al.* (1995) *Cell* 83, 1263-1271).

30 Evidence for EPO induced receptor dimerization is based primarily on constitutively active EPO-R mutants, which contain point mutations introducing cysteine substitutions into the extracellular domain at amino acid positions R129, E132, and E133 (Longmore *et al.*
33 (1991) *Cell* 167, 1089-102; Yoshimura *et al.* (1990) *Nature* 348, 647-649; Watowich *et al.*

(1992) *Proc Natl Acad Sci USA* 89, 2140-2144; Watowich *et al.* (1994) *Mol Cell Biol* 14, 3535-3549; Longmore *et al.* (1994) *Mol Cell Biol* 14, 2267-2277). The EPO-R mutants form
3 disulfide-linked homodimers in the endoplasmatic reticulum and on the cell surface
(Watowich *et al.* (1992)). Based on sequence alignments with the related GH receptor, these
mutations are expected to be in the receptor-dimer interface region. Expression of the
6 constitutively active EPO-R (R129C) mutant in BaF3 cells results in factor-independent
proliferation, and expression in primary cultures of mouse fetal liver cells induce
EPO-independent erythroid differentiation (Pharr *et al.* (1993) *Proc Natl Acad Sci USA* 90,
9 938-942). Furthermore, mice infected with a retrovirus carrying the EPO-R (R129C) mutant
develop erythroleukemia (Longmore *et al.* (1994)). Truncated signal transduction inactive
receptor mutants lacking part of the intracellular signaling domain are dominant-negative
12 when coexpressed with wild-type EPO-R (Watowich *et al.* (1994); Barber *et al.* (1994) *Mol
Cell. Biol* 14, 2257-2265). Both wild-type and truncated receptors can be
coimmunoprecipitated with an antibody directed against the C-terminus of the wild-type
15 receptor, which is not present in the truncated form (Miura *et al.* (1993) *Arch Biochem
Biophys* 306, 200-208), further suggesting the presence of receptor dimers on the cell surface.

Although dimerization of EPO-R is required, it is not sufficient for complete activation
18 of cells. Other accessory cellular factors may be required to send a proliferation signal and,
furthermore, these factors may be different from those required to send a differentiation
signal. It is desirable to identify molecules other than EPO that activate the EPO-R and
21 stimulate erythropoiesis and this invention meets that need.

SUMMARY OF THE INVENTION

24 An aspect of this invention is an antibody which activates erythropoietin receptors.

A second aspect of this invention is a bivalent molecule which activates erythropoietin
receptors, which bivalent molecule contains at least one binding domain that selectively
27 recognizes an epitope located on the extracellular domain of an erythropoietin receptor, which
epitope is selectively recognized by the monoclonal antibody produced by Hybridoma
#34.10.1 deposited at the ATCC as ATCC HB-12088.

30 A third aspect of this invention is a hybridoma cell line which produces a monoclonal
antibody which activates erythropoietin receptors, which monoclonal antibody contains two
binding domains that selectively recognizes an epitope located on the extracellular domain of

an erythropoietin receptor, which epitope is selectively recognized by the monoclonal antibody produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.

3 A fourth aspect of this invention is a method of treating a condition in a mammal in which diminished erythropoietin receptor activity contributes to the pathology and/or symptomatology of the condition, which method comprises administering to such animal a
6 therapeutically effective amount of an antibody which activates erythropoietin receptors.

A fifth aspect of this invention is method of treating a condition in a mammal in which diminished erythropoietin receptor activity contributes to the pathology and/or
9 symptomatology of the condition, which method comprises administering to such animal a therapeutically effective amount of a bivalent molecule which activates erythropoietin receptors, which bivalent molecule contains at least one binding domain that selectively
12 recognizes an epitope located on the extracellular domain of an erythropoietin receptor, which epitope is selectively recognized by the monoclonal antibody produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.

15 A sixth aspect of this invention is pharmaceutical composition comprising a therapeutically effective amount of an antibody which activates erythropoietin receptors in combination with a pharmaceutically acceptable excipient.

18 A seventh aspect of this invention is pharmaceutical composition comprising a therapeutically effective amount of a bivalent molecule which activates erythropoietin receptors, which bivalent molecule contains at least one binding domain that selectively
21 recognizes an epitope located on the extracellular domain of an erythropoietin receptor, which epitope is selectively recognized by the monoclonal antibody produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.

24

BRIEF DESCRIPTION OF THE DRAWINGS

27 The invention now being generally described, the same will be better understood by reference to the following detailed description of specific embodiments in combination with the figures that form a part of this specification.

30 FIG. 1 is a BIAcore sensogram recording and a graph of the data contained within the sensogram showing the binding kinetics of MAb34. A: BIAcore (Pharmacia Biosensor) sensograms of various concentrations of EPObp (ranging from 10 to 1500 nM) injected over
33 immobilized MAb34, corrected by data from control surfaces. Data from 8 representative

EPObp concentrations from a total of 16 are shown. B: The data were used for affinity determinations by plotting steady state values (ΔR_{eq}) versus EPObp concentrations. The solid line represents a fit of the data to the steady state model described by Karlsson *et al.* (Karlsson *et al.* (1991) *J. Immunol Methods* 145, 229-240).

FIG. 2 provides graphs showing MAb34-induced proliferation of BaF3/EPO-R cells and UT-7/EPO cells. A: Dose-dependent MAb34-induced proliferation of BaF3/EPO-R cells. Cells were incubated in the presence of [3 H]thymidine and various concentrations of MAb34 (open squares) and Fab34 (open circles) as described in Example 4. B: Dose-dependent cell proliferation of UT-7/EPO in the presence of various concentrations of MAb34 (open squares), control anti-Axl antibody unrelated to EPO (solid circles), and EPO (open triangles), respectively as described in Example 4. Experiments were done in duplicate.

FIG. 3 provides graphs showing MAb34-mediated proliferation and differentiation. Calculated values for K_A and K_D are given. Baseline values for proliferation ([3 H]thymidine incorporation in the absence of MAb34) were subtracted. A: Proliferation of BaF3/EPO-R cells. B: Proliferation of UT-7/EPO cells. C: Differentiation of CD34⁺ cells.

DESCRIPTION OF SPECIFIC EMBODIMENTS

EPO stimulates proliferation and differentiation of erythroid progenitor cells. Homodimerization of EPO-R by EPO on the cell surface is believed to be the key event in receptor activation and subsequent signal transduction (Youssoufian *et al.*). Bivalent molecules that possess two identical binding sites for a given region on the extracellular domain of EPO-R, such as antibodies, for example, can bind two EPO-R molecules thereby bringing them into close proximity to permit dimerization. These bivalent molecules must bind so as to activate the signal transduction pathway and it is desirable that such activation result in both proliferation and differentiation of erythroid progenitors. It is also possible for the binding domains of the bivalent molecules to recognize different regions of EPO-R and have a similar or an enhanced effect. Such bivalent molecules are useful for researching the mechanism of EPO-R activation and signal transduction and have therapeutic and diagnostic applications as well.

Definitions

"Purified" means that the monoclonal antibody is separated from at least some of the proteins normally associated with the monoclonal antibody and preferably separated from all cellular materials other than proteins.

"Epitope" means a region of an EPO-R recognized by a first antibody wherein the binding of the first antibody to the region prevents binding of a second antibody or other bivalent molecule to the region. The region encompasses a particular core sequence or sequences selectively recognized by a class of antibodies and can include additional EPO-R sequences. For example, several antibodies can selectively bind to the sequence XYZ and although they may bind with differing affinities to abcXYZ than to XYZdef, they are nevertheless binding to the same epitope. The epitope can be contiguous or non-contiguous such that the first antibody selectively recognizes a peptide fragment containing the core sequence XYZ, but the second antibody or other bivalent molecule selectively recognizes a fragment containing the core sequence only in its native conformation on the EPO-R. For example, the tertiary structure of the EPO-R can give rise to the epitope pqrXYZ from the primary sequence of pqr.....XYZ. An antibody specific for pqrXYZ would not be expected to bind the peptide pqr.....XYZ as effectively as it would the EPO-R itself, yet it will selectively recognize either the peptide fragment or the EPO-R. Thus, abcXYZ, XYZdef, and pqrXYZ are all the same epitope because they share the same core sequence. The core sequence comprises at least 3 amino acid residues, preferably 4-20 amino acid residues, and can comprise as many as 35 amino acid residues. Thus, the "MAb34 epitope" is an epitope that is selectively recognized by the MAb34 antibody described in more detail below.

"Selectively recognizes" or "selectively recognized" means that binding of the antibody or other bivalent molecule to an epitope is at least 2-fold greater, preferably 2-5 fold greater, and most preferably more than 5-fold greater than the binding of the bivalent molecule to an unrelated epitope or than the binding of an unrelated bivalent molecule to the epitope, as determined by techniques known in the art and described herein, such as, for example, ELISA and cold displacement assays.

"Bivalent molecule" or "BV" means a molecule capable of binding to two separate erythropoietin receptors at the same time, thereby forming a molecular complex. The bivalent molecule is not limited to having two and only two binding domains and can be a polyvalent molecule or a molecule comprised of linked monovalent molecules so long as at least two EPO-R binding domains are provided. The binding domains of the bivalent

molecule can selectively recognize the same epitope or different epitopes located on the EPO-R extracellular domain, so long as at least one binding domain selectively recognizes an epitope recognized by MAb34 (i.e., the MAb34 epitope) and the resultant (EPO-R)₂:BV complex is active in signal-transduction. The bivalent molecule can be proteinaceous or non-proteinaceous provided that it can form a complex sufficiently stable to activate the EPO-R or to be detected. The binding domains can be linked in any of a number of ways including, but not limited to, disulfide bonds, peptide bridging, amide bonds, and other natural or synthetic linkages known in the art (Spatola *et al.*, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J.S., "Trends Pharm Sci" (1980) pp. 463-468 (general review); Hudson *et al.*, *Int J Pept Prot Res* (1979) 14, 177-185; Spatola *et al.*, *Life Sci* (1986) 38, 1243-1249; Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314; Almquist *et al.*, *J Med Chem* (1980) 23, 1392-1398; Jennings-White *et al.*, *Tetrahedron Lett* (1982) 23, 2533; Szelke *et al.*, European Application EP 45665; Chemical Abstracts 97, 39405 (1982); Holladay, *et al.*, *Tetrahedron Lett* (1983) 24, 4401-4404; and Hruby, V.J., *Life Sci* (1982) 31, 189-199).

"Proteinaceous" means the molecule referred to comprises a polypeptide sequence. Thus a proteinaceous molecule can be comprised entirely of polypeptide sequences or it can comprise one or more non-peptidic substituents in addition to polypeptide sequences.

"Therapeutically effective amount" means an amount that provides a therapeutic effect for a given condition and administration regimen. In the present invention, the therapeutic effect is an increase in erythrocyte levels, which can be evidenced by a rise in hematocrit in the patient being treated.

"Treating", as in treating a condition, means (1) preventing the condition from occurring in a mammal which may be predisposed to the condition but does not yet experience or display symptoms of the disorder, (2) inhibiting the condition (e.g., arresting development of the condition) or (3) ameliorating the condition (e.g., causing regression of the disorder).

"Pathology of a condition" means the essential nature, causes and development of the condition as well as the structural and functional changes that result from the disease processes.

“Symptomatology of a condition” means any morbid phenomenon or departure from the normal in structure, function or sensation experienced by the patient and indicative of the condition, their production and the indications they furnish.

In one aspect of the invention an antibody is provided that selectively recognizes the extracellular domain of the erythropoietin receptor to form a molecular complex active in signal transduction. In particular, it should be noted that the invention is not limited to use or identification of the specific monoclonal described in the Examples section below. Any antibody that selectively recognizes an epitope and that results in homodimerization of the erythropoietin receptor and subsequent cell proliferation and differentiation of erythroid precursors is encompassed by the present invention. Use of such antibodies is easily accomplished by one of ordinary skill in the art given the teachings of the present specification.

In another aspect of the invention a bivalent molecule (BV) is provided that selectively recognizes the extracellular domain of the erythropoietin receptor. The bivalent molecule can bind the extracellular domains of two erythropoietin receptor to form a molecular complex active in signal transduction. In particular, it should be noted that the invention is not limited to use or identification of the specific monoclonal described in the Examples section below. Any bivalent molecule as defined above that selectively recognizes an epitope selectively recognized by monoclonal antibody MAb34 (MAb34 epitope) and that results in homodimerization of the erythropoietin receptor and subsequent cell proliferation and differentiation of erythroid precursors is encompassed by the present invention. Use of such molecules is easily accomplished by one of ordinary skill in the art given the teachings of the present specification.

The bivalent molecule can have a detectable label attached thereto, such as a fluorescent label (e.g., fluorescein, isothiocyanate (FITC)), an affinity label (e.g., biotin), an enzymatic label (e.g., horseradish peroxidase or alkaline phosphatase), or an isotopic label (e.g., ^{125}I) or any other such detectable moiety. Apart from the ability of the bivalent molecules of the invention to modulate erythroid precursor proliferation and differentiation, these molecules are also useful for the detection of the presence or absence of erythropoietin receptors in a sample. A preferred bivalent molecule is an antibody that selectively recognizes the MAb34 epitope located on the extracellular domain of the erythropoietin receptor. Antibodies can be produced by the immunization of various animals, including mice, rats, rabbits, goats, primates, humans and chickens with EPO-R or peptide fragments

of EPO-R containing the MAb34 epitope. Preferably, the protein is purified prior to immunization of the animal. The EPO-R can be purified by methods known in the art, for example, gel filtration, ion exchange, affinity chromatography, etc. The EPO-R can be naturally occurring or genetically engineered as is apparent to one of ordinary skill in the art. Preferably the antibody is purified. Affinity chromatography or any of a number of other techniques known in the art can be used to isolate polyclonal or monoclonal antibodies from serum, ascites fluid, or hybridoma supernatants.

The antibody can be of the isotypes IgA, IgE, IgM, preferably is an IgG antibody, and can be an IgD antibody, although this is less preferred. IgG subclasses include IgG1, IgG2a, IgG2b and IgG3 in the mouse; IgG1, IgG2a, IgG2b, and IgG2c in the rat; and IgG1, IgG2, IgG3 and IgG4 in the human. Useful antibodies of the invention are chosen upon consideration of such factors as complement fixation, autoaggregation, ability to mediate mast cell degranulation, ability to bind Fc receptors or macrophage receptors and resistance to proteolytic enzymes. Preferably, the bivalent molecules of the invention are resistant to proteolytic cleavage and are not excessively immunogenic. Also included are fully human antibodies selectively recognizing the MAb34 epitope produced in genetically altered mice (PCT Application No. 93/122227).

Preferably, the antibody is a monoclonal antibody. Methods of generating monoclonal antibodies are known in the art and are described in detail, for example, by Oi and Herzenberg in "Selected Methods in Cellular Immunology" (1979) (B.B. Mishell and S.M. Shiigi, eds.), San Francisco: W.J. Freeman Publishers, pp. 351-352. In short, after immunization of an animal spleen cells are isolated and fused with myeloma cells, such as mouse myeloma cell lines P3X63Ag8.653 (ATCC CRL 1580); P3-NS1/1Ag 4; and S194/5, XXO, BUI; human fusion partners such as UV 729-6 and SKO-007; and mouse-human hetero-myeloma lines, such as SHM-A6 and SHM-D33 (PCT Application No. 81/00957; Schlom *et al.*, *Proc. Natl. Acad. Sci. USA* (1980) 77, 6841-6845; Croce *et al.* (1980) *Nature* 288, 488-489). The fusion is carried out in the presence of a non-ionic detergent for a short period of time and removed. The cells are then subjected to selective conditions, such as HAT and ouabain, for example, that are cytotoxic to the parent cells, but not fused hybrid cells. Such hybridoma cell lines are also encompassed by the invention and provide an inexhaustive supply of monoclonal antibody. Typically, antibody titers are determined by methods such as enzyme linked immunosorbent assays (ELISA) (Engrall (1977) *Med. Biol.* 55, 193-200) to determine their affinity for their binding partners. The ability of the

antibodies to activate EPO-R can be determined by cell proliferation or differentiation assays, some of which are described herein.

3 A preferred monoclonal antibody is MAb34, which is deposited at the ATCC as Hybridoma #34.10.1 assigned ATCC HB-12088. The monoclonal antibody Mab34 directed against the extracellular domain of the EPO-R that activates EPO-R by dimerization and thus
6 mimics EPO action as is demonstrated below in Example 4. This bivalent IgG antibody triggers the proliferation of EPO-dependent cell lines and induces differentiation of erythroid precursors *in vitro*. Activation of cell proliferation and differentiation show a bell-shaped
9 response curve over the range of antibody concentration tested with a maximum occurring at MAb34 concentrations in close vicinity to its K_D . Indeed, a mathematical model (Perelson, A.S. (1984) in *Cell Surface Dynamics: Concepts and Models* (Perelson, A.S., DeLisi, C., and Wiegel, F.W., eds), pp. 223-276, Marcel Dekker, Inc., New York, NY) predicts that
12 a maximum of 2:1 receptor/antibody complexes is formed at a concentration of $0.5 \times K_D$.

The mechanism of receptor activation by homodimerization implies that at high
15 concentrations the formation of 1:1 receptor/ligand complexes is favored over 2:1 complexes, thereby turning the ligand agonist into an antagonist. Thus, EPO and MAb34 should self-antagonize at high concentrations in both cell proliferation and differentiation assays.
18 Example 4 demonstrates that EPO and MAb34 antagonize ligand-dependent cell proliferation with IC_{50} concentrations of approximately $20 \mu M$ and $2 \mu M$, respectively. We have further analyzed the MAb34:EPO-R interaction using a mathematical model describing antibody
21 mediated receptor dimerization. The predicted receptor dimer formation on the cell surface was consistent with the observed proliferation and differentiation activity data.

Although one of ordinary skill in the art would expect that all bivalent antibodies
24 specific for the extracellular domain should dimerize the receptor, the vast majority of antibodies specific to the EPObp in our screen are not active (47 out of 48) and form inactive complexes (see Example 2). Surprisingly, MAb34 does not bind to the EPO binding site of
27 the erythropoietin receptor as determined by competition with EPO in a cold displacement assay. Thus, the bivalent molecules of the invention have revealed a new site of erythropoietin receptor interaction not previously known to trigger receptor activation and
30 signal transduction.

Another preferred monoclonal antibody is a high-affinity variant of MAb34. Such variants can be prepared by a number of methods known in the art or developed in the future,
33 including the random or site-directed mutagenesis of the variable region of MAb34. For

example, degenerate oligonucleotides can be substituted for the variable region in MAb34 and the resultant antibody population screened by competition with MAb34 for EPO-R binding.

3 As described further below, phage display can also be utilized for this purpose.

The antibody can be a chimeric antibody such as a humanized antibody or a CDR-grafted antibody in which regions other than the binding domains are replaced with
6 human immunoglobulin polypeptide sequences (US Patent Nos. 4,816,567 and 5,225,539). Another preferred chimeric antibody is a bispecific antibody in which one of the binding domains selectively recognizes an epitope located on the extracellular domain of EPO-R other
9 than the MAb34 epitope, provided that the chimeric antibody can form an (EPO-R)₂:antibody complex active in signal transduction, a property possibly dependent upon the distance and geometry of the receptors.

12 Another preferred aspect is a bivalent proteinaceous molecule other than an antibody comprising two binding domains that selectively recognize the MAb34 epitope. For example, peptides can be designed based on an analysis of the amino acid sequence of the
15 complementarity-determining region (CDR) of MAb34. A bivalent molecule can be synthesized containing two or more of such peptides linked or fused to each other and possibly to an additional polypeptide structure provided as a scaffold for stability or some
18 other purpose such as, for example, the recruitment of a tertiary molecule. Alternatively, random peptide libraries prepared by techniques known in the art, such as, for example, peptide chemistry, phage display (Ladner et al; Huse; Devlin; Dower *et al.*; Markland *et al.*)
21 or the yeast two-hybrid system can be screened by competitive inhibition of MAb34 binding to EPO-R to identify a polypeptide sequence that selectively binds to the MAb34 epitope. This peptide can then be synthesized in a bivalent form and tested for an ability to activate
24 EPO-R by any of the methods herein described, known in the art, or developed in the future.

The bivalent proteinaceous molecule can be a bispecific molecule in which one of the binding domains selectively recognizes an epitope located on the extracellular domain of
27 EPO-R other than the MAb34 epitope, such as, for example, an epitope corresponding to the EPO-binding domain. The bispecific molecule can be a bispecific antibody as mentioned above, a chimeric molecule comprising an immunoglobulin portion and a
30 non-immunoglobulin portion, or an entirely non-immunoglobulin proteinaceous bispecific molecule, so long as the bispecific molecule is capable of forming an active molecular complex with EPO-R.

Another preferred aspect is a non-proteinaceous bivalent molecule. Such a molecule can be identified and produced by techniques known in the art, such as, for example, 3 peptidomimetics. Such mimetics can be produced by rational drug design based on molecular modeling and the polypeptide sequence of the CDR of MAb34. Alternatively, combinatorial chemical libraries can be screened by competitive inhibition of MAb34 binding to EPO-R to 6 identify a compound that selectively binds to the MAb34 epitope and then the compound can be synthesized in a bivalent form and tested for its ability to form an active (EPO-R)₂:BV complex by any of the methods herein described.

9 Bivalent molecules selectively recognizing the MAb34 epitope can be produced in a number of methods using techniques known in the art. These methods are not part of the invention but are provided herein for the convenience of the reader. One method is initially 12 to screen a population of candidate molecules for an ability to compete with MAb34 for binding of EPO-R, and then to perform a functional determination of an ability to activate EPO-R, such as, for example, thymidine uptake proliferation assays, erythroid cell 15 differentiation assays, or any assays exploiting steps in the signal transduction cascade. Suitable assays are provided for illustrative purpose in the Examples section. Another method is to synthesize peptides corresponding to the MAb34 epitope for use as a binding 18 partner in binding assays. The MAb34 epitope can be mapped by preparing overlapping synthetic peptides that span the extracellular domain EPO-R and screening them for binding to MAb34. Another procedure that can be used for mapping the MAb34 epitope is phage 21 display (Ladner *et al.*, PCT Application No. PCT/US92/01456; Huse, PCT Application No. PCT/US91/07141; Devlin, PCT Application No. PCT/US91/03332; Dower *et al.*, PCT Application No. PCT/US91/02989 and PCT/US91/04384; Markland *et al.*, PCT Application 24 No. PCT/US92/01539) whereby bacteriophage displaying random peptide sequences on the phage surface are screened by binding to immobilized MAb34 and the peptide sequences are subsequently deduced from the polynucleotide sequence obtained from nucleic acid 27 sequencing.

The invention also provides an (EPO-R)₂:BV complex. Such a complex, when present on the surface of a cell, is useful for modulating proliferation and differentiation of erythroid 30 precursor cells. Such a complex, whether present on the surface of a cell or isolated from other cellular components, can also be used for EPO-R activator drug discovery where compounds that are potential candidate drugs for the treatment of disorders treatable by 33 EPO-R activation are screened as described above for their ability to competitively inhibit

complex formation between EPO-R and previously identified bivalent molecules of the invention.

3 In another preferred embodiment, the invention provides methods and kits for
detecting the presence or absence of an erythropoietin receptor or an activatable EPO-R in
a biological sample. In a preferred aspect, the detection is achieved by a binding assay in
6 which the level of binding of a bivalent molecule to EPO-R is detected. Basically, the
biological sample is contacted with the bivalent molecule and the level of binding is detected.
Many similar assays are well known in the art and include, but are not limited to, ELISA's,
9 binding displacement assays, binding competition assays, BIAcore (Pharmacia Biosensor)
analysis, radioimmunoassays, enzyme-linked immunoassays, Western immunoblot assays and
the like. The biological samples include tissue specimens, intact cells, or extracts thereof.
12 Antibodies may be used as part of a diagnostic kit to detect the presence of EPO receptors
in a biological sample. Such kits employ bivalent molecules, preferably a monoclonal
antibody, having an attached label to allow for detection.

15 The detection can also be achieved by a functional assay in which it is the activation
of the receptor that is detected. For example, any of a number of cellular products of the
signal transduction cascade triggered by EPO-R activation can be monitored in a functional
18 assay, such as nitric oxide, prostaglandins, cAMP, cGMP, Ca^{2+} , inositol phosphates and the
like. Alternatively, kinase and phosphatase activities resulting from EPO-R activation can
also be monitored in a functional assay. Such methods can be used in the diagnosis of
21 anemia and other diseases characterized by a dysfunctional EPO-R or otherwise related to
EPO-R activation.

The invention also provides methods of modulating the endogenous activity of an
24 erythropoietin receptor in a mammal. Typically activation of the EPO-R in an erythroid
precursor cell results in cell proliferation or differentiation. Such methods are useful for
treating disorders characterized by low erythrocyte levels. Thus, any condition treatable with
27 EPO, such as anemia, for example, is amenable to treatment with the bivalent molecules of
the invention. Any of the bivalent molecules of the invention can be used for this purpose,
subject to such considerations as variations in bioavailability, antigenicity, and potency among
30 the different bivalent molecules. For example, MA34 may be more potent than another
antibody, but for long-term use, a slightly less potent humanized antibody may be preferred.
Other non-immunoglobulin bivalent molecules may be preferred where stability or the need
33 for an orally active drug is an issue. The amount and method of administering the

therapeutic molecules of the invention can be ascertained by one skilled in the art. Preferably, administration of large bivalent molecules is by injection, whether subcutaneous, intramuscular, or intravenous. Smaller, less peptidic bivalent molecules can be administered orally as well as intravenously.

The invention also provides a pharmaceutical composition comprising a therapeutically effective amount of the bivalent molecule of the invention in a pharmaceutically acceptable adjuvant, which can be selected from one or more of a diluent, carrier, preservative, emulsifier, anti-oxidant and/or stabilizer. Pharmaceutically acceptable adjuvants are known to one skilled in the art (Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1990)).

EXAMPLES

3 The invention now being generally described, the same will be better understood by
reference to the following detailed examples, which are provided for the purpose of
illustration only and are not to be considered limiting of the invention unless otherwise
6 specified.

EXAMPLE 1: GENERATION OF EPO-R MONOCLONAL ANTIBODIES

9 In order to obtain an antibody capable of dimerizing an EPO-R, monoclonal antibodies
were raised against the extracellular, ligand binding domain of the human EPO-R
(EPObp).

12 Expression and affinity purification of soluble human EPO-R:

DNA encoding a soluble truncated EPObp was generated by the polymerase chain
reaction (PCR) using the full-length cDNA as template. The amplification product introduces
15 a TAG termination codon 5' of the transmembrane region and encodes the extracellular
domain comprising amino acids 1 through 249 of the published sequence (Jones *et al.* (1990)
Blood 76, 31-35). The PCR product was subcloned into expression vector pRc/CMV
18 (Invitrogen) and stably transfected into CHO cells. Individual clones secreting EPObp were
selected by limiting dilution cloning. Roller bottles (surface area 1,700 cm², Corning,
Corning, NY) were seeded with the stable cell line and grown to confluence in RPMI plus
21 10% FBS. Cells were washed twice in serum free RPMI medium and cultured in 200 ml of
serum-free RPMI. Cell supernatant was collected after two days and fresh medium was added
for another two days. The EPObp was secreted at an approximate concentration of 0.2 µg/ml
24 and was purified by EPO affinity chromatography.

EPO was oxidized with 10 mM NaIO₄ and biotinylated using 10 mM biotin hydrazide
(Pierce) following the manufacturer's instructions. A ligand affinity column was prepared by
27 immobilizing biotinylated EPO (10 mg) on Streptavidin 3M Emphaze beads (3 ml; Pierce),
overnight in Dulbecco's phosphate buffered saline (PBS, Irvine Scientific) at 4°C. The beads
were separated from the supernatant by centrifugation, and incubated with 10 mM biotin in
30 PBS for 2 h at 4°C to saturate all biotin binding sites. After washing with PBS, the EPO
coated beads were packed in a glass column (Omnifit). Cell supernatant (10 L) was
concentrated and diafiltered to 1 L in 20 mM Tris HCl, pH 7.6 and loaded on the column
33 at a flow rate of 0.7 ml/min. The column was washed with 50 ml of 20 mM Tris/HCl, pH

7.6. Bound EPObp was eluted with 750 mM NaCl in 20 mM Tris/HCl, pH 7.6. SDS-PAGE analysis showed a single 30 kD EPObp band. The EPObp fractions were pooled, concentrated and buffer exchanged with PBS to a final concentration of 0.8 mg/ml.

Generation of monoclonal antibodies and screening for binding to EPObp:

Monoclonal antibodies were generated essentially as described in Galfré *et al.* (1981) *Methods. Enzymol.*, 73, 3-46). Five Balb/c mice were immunized by seven subcutaneous injections at two sites over a period of 14 weeks. Each 50 µl injection contained 25 µg EPObp in Freud's adjuvant.

Antibody titers were measured by ELISA after 12 weeks. Polysorb microtiter plates (Nunc, Roskilde, Denmark) were incubated with 5 µg/ml EPObp in PBS for 1 h at 37°C, washed and then blocked with 20 mg/ml bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serial dilutions of sera samples in PBS, 1 mg/ml BSA, 0.05% Tween-20 were added and incubated for 1 h at 37°C. After washing, the plates were incubated with a sheep anti-mouse IgG coupled to horseradish peroxidase (HRP; Sigma, St. Louis, MO) at 0.1 ng/ml in PBS containing 1 mg/ml BSA, 0.02% Tween-20 for 1 h at 37°C. Plates were then washed and 100 µl TMB/H₂O₂ developing solution (Pierce, Rockford, IL) was added and incubated for 5 min. Color development was stopped by adding 100 µl 1M sulfuric acid, and the OD₄₅₀₋₆₅₀ was determined (Molecular Devices). The titers ranged from 10,000 to 50,000.

A final injection of 150 µg EPObp was given to the mouse expressing the highest antibody titer. After 3 d, spleen cells were isolated and fused with myeloma strain P3XAg8.653 (ATCC CRL 1580) to generate 475 hybridomas. After selection in HAT medium (Littlefield (1964) *Science* 145, 709-710) for 10 days, supernatants were screened for specific antibody production by ELISA.

Because a low number of agonist antibodies is expected, two different methods for immobilization of the EPObp were used to ensure identification of a maximum number of EPObp MAb's. In ELISA #1, EPObp was covalently immobilized. EPObp was oxidized in 1 mM NaIO₄, 50 mM sodium acetate, pH 5.5 at 4 °C for 30 min in the dark. The protein was separated from periodate on a NAP-5 column (Pharmacia-Biotech Inc., Piscataway, NJ) and incubated on hydrazide-activated microtiter plates (Unisyn, San Diego, CA) at 2 µg/ml (100 µl per well) for 1 h at room temperature. Plates were blocked with PBS, 20 mg/ml BSA for 1 h. In ELISA #2 EPObp was immobilized via MAb 2E12, a specific, non-neutralizing rat monoclonal antibody directed against EPObp: Polysorb microtiter plates were incubated with 10 µg/ml MAb 2E12 for 1 h at 37°C, washed and blocked with PBS

containing 20 mg/ml BSA for 1 h at 37°C. EPObp (1 µg/ml) was added in PBS, 1 mg/ml BSA, 0.02 % Tween-20 for 1 h at 37°C. After the immobilization of EPObp, both ELISA
3 protocols were identical. Hybridoma supernatants were added and incubated for 1 h at 37°C. After washing, bound antibodies were determined using anti-mouse IgG - HRP conjugate as described above. A total of 48 supernatants were found to bind to immobilized EPObp in
6 at least one out of the two ELISA assays and were further screened for agonist activity.

EXAMPLE 2: SCREENING OF EPO-R MONOCLONAL ANTIBODIES FOR RECEPTOR AGONIST 9 ACTIVITY

To identify a monoclonal antibody capable of inducing cell proliferation, positive clones were transferred to 24-well microtiter plates, and supernatants were assayed in a
12 thymidine uptake proliferation assay using the cell line BaF3/EPO-R and the parental BaF3 as a control. BaF3 cells transfected with a full-length human EPO-receptor can grow in media containing either IL-3 or human EPO.

15 Development of an EPO-Dependent BaF3/EPO-R Cell Line:

An EPO-dependent BaF3/EPO-R cell line was generated by transfecting the full-length human EPO receptor into BaF3 cells, a murine IL-3 dependent cell-line (Palacios *et al.*
18 (1985) *Cell* 41, 727-734). A cDNA encoding the full-length human EPO receptor (Winkelmann *et al.* (1990) *Blood* 76, 24-30; Jones *et al.* (1990) *Blood* 76, 31-35) was cloned into plasmid expression vector pRc/CMV (Invitrogen, San Diego, CA). After electroporation
21 into BaF3 cells, the cells were cultured in RPMI 1640 medium containing IL-3 for 2 days. Cells were washed twice, transferred into RPMI 1640 plus 135 pM EPO, and selected for EPO-dependent growth. Individual clones were selected by limited dilution cloning. The
24 EPO-dependent cell line chosen for this study proliferates in the presence of EPO with an EC₅₀ of 15 pM. Scatchard analysis revealed 800 receptors per cell binding EPO with 300 pM affinity, assuming single site binding. Cells were maintained in RPMI 1640 with 10% fetal
27 bovine serum (FBS), 20 mM HEPES pH 7.8, and 10 mM mercaptoethanol supplemented with 100 pM EPO. BaF3 cells were supplemented with 10% IL-3 containing WEHI-3B conditioned medium.

30 Thymidine uptake proliferation assays:

BaF3/EPO-R and IL-3-dependent BaF3 parental cells were incubated with individual hybridoma supernatants in the absence of EPO and IL-3, respectively, and proliferation was
33 measured by [³H]thymidine incorporation. BaF3 and BaF3/EPO-R cells were grown to late

logarithmic phase, collected by centrifugation, washed three times with RPMI 1640 media (containing 10% FBS and 10 mM HEPES pH 7 in the absence of EPO and IL-3), then
3 starved in the same media for 2 hours. Antibody test samples (hybridoma supernatants or purified proteins) were diluted at least 4-fold into 100 μ l media and 100 μ l cells were added (25,000 cells per well). EPO was dialyzed against 10 mM HEPES pH 7.0, and 100 μ l test
6 samples were combined with 100 μ l cells (25,000 cells per well) in 2-fold concentrated medium. Plates were incubated for 4 hours at 37°C and 5% CO₂ in a humidified tissue culture incubator. Then 0.5 μ Ci [³H]thymidine (Amersham), diluted into 20 μ l medium, was
9 added and incubation continued for another 15 hours. Cells were harvested onto glass fiber filtermats using a Tomtec cell harvester (Wallac Oy), and incorporated radiolabel was determined using a Microbeta 1450 scintillation counter (Wallac, Turku, Finland).

12 The hybridoma supernatant of MAb clone 34 stimulated thymidine uptake in BaF3/EPO-R cells but not in BaF3 cells, indicating that the proliferation was specific to the presence of EPO-R. Hybridoma clone MAb34 was subcloned twice by limiting dilution.
15 Ig-isotyping was performed using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit from Boehringer Mannheim (Indianapolis, IN). MAb34 is an IgG monoclonal which was subtyped as IgG α 1.

18

EXAMPLE 3: PURIFICATION AND DETERMINATION OF BINDING CHARACTERISTICS OF AN EPO-R AGONIST

21 To further characterize the binding characteristics of MAb 34 for an erythropoietin receptor, MAb34 was purified and used in cold displacement binding assays. Binding kinetics were examined using the BIAcore system. MAb34 was purified by protein G affinity
24 chromatography, and Fab fragments (Fab34) were prepared by papain cleavage. 0.75 μ g and 1.5 μ g of EPObp was heat-denatured and analyzed by reducing SDS-PAGE on a 12% acrylamide gel and subsequent transfer to nitrocellulose. The blot was incubated with MAb34
27 (10 μ g/ml) and subsequently with anti-mouse IgG coupled to horseradish peroxidase. A robust signal was obtained for both 0.75 μ g and 1.5 μ g samples of EPObp. This immunoblot analysis of heat-denatured and reduced EPObp suggested that MAb34 recognizes a linear
30 continuous epitope.

Purification of Mab34:

Hybridomas were grown in 47.5% RPMI, 47.5% DMEM, 5% FBS. Culture
33 supernatant was filtered through a 0.2 μ m membrane. A 6 ml protein G Sepharose 4 fast flow

column (Pharmacia) was packed at 80 psi pressure. A 1 l sample was loaded at 4 ml/min at 4°C, followed by washing with >5 column volumes of PBS. MAb34 was then eluted from
3 the column with ImmunoPure IgG elution buffer (Pierce) at 2 ml/min. The eluate was immediately neutralized to pH 7.5 by adding 3 M Tris. The purity was evaluated by non-reducing SDS-PAGE. F_{ab} fragments were generated by papain cleavage using
6 ImmunoPure IgG1 F_{ab} Preparation Kit (Pierce) following the manufacturer's instructions. The F_{ab} was further purified by gel filtration, using a Superdex 75 column (1.6 cm x 60 cm, Pharmacia), eluted with PBS, and analyzed for purity by SDS-PAGE.

9 **EPO cold displacement assay:**

OCIM1 cells, a human erythroleukemia cell line which expresses EPO-R on the cell surface (Broudy *et al.* (1988) *Proc. Nat. Acad. Sci. USA* 85, 6513-6517), were grown in
12 IMDM, 10% FBS, 1% penicillin-streptomycin-fungisone to approximately 2-5 X 10⁵ cells/ml. Cells were collected by centrifugation, washed two times in binding buffer (RPMI 1640, 1% BSA, 25 mM HEPES pH 7.3), then resuspended in binding buffer containing 0.1%
15 sodium azide and 10 µg/ml cytochalasin B at 1-2 X 10⁷ cells/ml. Cells (100 µl) in 96 well tissue culture plates were then incubated with 10 µl sample and 10 µl [¹²⁵I]-EPO (Amersham high specific activity; 3000 Ci/mmol, 2 mCi/ml) in a humidified tissue culture incubator at
18 37°C. After 3 h, cells were centrifuged through phthalate oil (60:40 (v/v) dibutyl/dinonyl phthalate) in titer tubes. The tubes containing cell pellets were quick frozen in a dry ice-ethanol bath and the cell pellet was clipped and then counted in a LKB 1277
21 gammamaster automatic gamma counter (Pharmacia).

MAb34 did not compete with [¹²⁵I]-EPO for binding to EPO-R in a displacement assay using human OCIM1 cells (Broudy *et al.* (1988) *Proc. Nat. Acad. Sci. USA* 85, 6513-6517),
24 indicating that the binding sites of EPObp for EPO and for MAb34 are different.

Analysis of MAb34 and Fab34 binding kinetics:

The binding kinetics of both the MAb34 and Fab34 to the EPObp were characterized
27 using surface plasmon resonance. For MAb34 kinetics the antibody was immobilized to avoid avidity effects. For Fab34 kinetics, we immobilized EPObp.

Kinetic parameters for the interaction of MAb34 and its Fab fragment (Fab 34) with
30 EPObp were measured by using real-time biospecific interaction analysis (BIAcore) (Johnsson *et al.* (1992) *Adv. Biosens.* 2, 291-336). The BIAcore system, CM-5 sensor chip, and reagents were from Pharmacia Biosensor, Piscataway, NJ. All injections on the sensor chip
33 surface were at a flow rate of 5 ml/min and 25 °C unless otherwise stated. Between injections

of reagents, the sensor chip was continuously washed with 10 mM HEPES, pH 7.5, 150 mM NaCl, 3.4 mM EDTA and 0.005 % surfactant P₂₀. The interaction of MAb34 with EPObp was characterized by coupling approximately 6800 resonance units (RU) of MAb34 to the sensor chip surface using standard amine immobilization chemistry (Johnsson *et al.* (1991) *Anal Biochem.* 198, 268-277). Samples of EPObp ranging in concentration from 10-1500 nM were injected for 7 minutes over the MAb34 surface and over a control flow cell. After each injection of EPObp, a 1 minute pulse of 1 M formic acid was used to regenerate the MAb34 surface. To measure the interaction of EPObp and Fab34, oxidized EPObp (app. 500 RU) was immobilized via carbohydrazide coupling to the carboxymethylated dextran matrix (Karlsson *et al.* (1991) *J. Immunol. Methods* 145, 229-240). Injections of Fab34 spanned a concentration range of 1-500 nM. After each injection of Fab34, the EPObp surface was regenerated with a 50 ml pulse, at 50 ml/min, of 10 mM CAPS pH 10.4. Transformation and analysis of data were performed with BIAevaluation software (Pharmacia Biosensor, Piscataway, NJ). Dissociation rate constants were determined by measuring dissociation of bound analyte in buffer flow. To minimize potential rebinding effects, only the initial 15 s of each dissociation profile was used for calculation of the dissociation rate constant.

Determinations of affinity were made from steady state equilibrium measurements and k_d was inferred from the relationship $K_D = k_d/k_a$. Although determination of dissociation rate constants (k_d) was straightforward using the BIAevaluation software provided by the manufacturer, direct measurements of association rate constants (k_a) were severely hindered by mass transport limitations. Hence, the steady state (R_{ss}) binding values were used to calculate the affinity of the interaction (FIG. 1) (Karlsson *et al.*). The affinity constants calculated for MAb34 and Fab34 are summarized in Table 1. Both bind to the EPObp with an affinity of approximately 50 nM.

TABLE 1

	immobilized molecule	analyte	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [nM]
3	Mab34	EPObp	3.2×10^5	0.017	54
6	EPObp	Fab34	4.6×10^5	0.02	43

Table 1. BIAcore analysis of MAb34 and Fab34 interactions with EPObp.

9 The values for k_d were determined from $K_D = k_d/k_a$. Standard errors in all cases were less than 10%.

12 EXAMPLE 4: EPO-R AGONIST STIMULATES PROLIFERATION IN BOTH RECOMBINANT AND NON-RECOMBINANT EPO-DEPENDENT CELL LINES

Purified MAb34 was tested for its ability to stimulate proliferation of both
15 recombinant and non-recombinant cell lines. Purified MAb34 was able to stimulate
proliferation in the EPO-dependent BaF3/EPO-R cell line using the procedure described in
Example 2. A dose dependent response evaluation in a [3H]thymidine uptake cell proliferation
18 assay revealed EC_{50} values of approximately 10 nM (FIG. 2A). The effect of MAb34 was
specific to EPO-R, because it did not stimulate growth of the parental BaF3 cell line. Based
on the maximal amount of [3H]thymidine incorporation, the potency of MAb34 was 8 to 10
21 -fold lower than the potency of EPO (FIG. 2A). In contrast to the bivalent MAb34,
monovalent Fab fragments did not stimulate proliferation of the BaF3/EPO-R cell line (FIG.
2A), although the affinities of MAb34 and Fab34 to EPObp were identical (Table 1).

24 The cell proliferation experiment was repeated using the EPO-dependent human
hematopoietic cell line UT-7/EPO (Komatsu *et al.* (1993) *Blood* 82, 456-464), which
expresses endogenous EPO-R. UT-7/EPO cells (Komatsu *et al.* (1993) *Blood* 82, 456-464)
27 and were grown in 1X Iscove's Modified Dulbecco's Medium (IMDM) with L-glutamine,
25 mM HEPES, 3.024 g/l sodium bicarbonate, 10% FBS, 1%
L-glutamine-penicillin-streptomycin solution (Irvine Scientific) containing 270 pM EPO.

30 UT-7/EPO cells were grown to approximately 3×10^5 cells/ml, collected by
centrifugation, washed twice with PBS and resuspended at 50,000 cells/ml in assay medium
(RPMI 1640 medium, 1% L-glutamine, 4% FBS). Test samples, 100 μ l diluted in assay
33 medium at least 5-fold, were added to wells. Then 50 μ l cells were added (5,000 cells per

well) and plates were incubated at 37°C and 5% CO₂. After 72 hours, 50 µl methyl-[³H]thymidine (1 mCi/ml; 20 Ci/mmol) diluted 1:100 in assay medium was added.

- 3 Cells were incubated for another 4 hours at 37°C and 5% CO₂. Labeled cells were harvested onto glass fiber filtermats using a PHD cell harvester (Cambridge Technology, Inc.). Filters were rinsed with 2-propanol, dried and counted in a Beckman Model LS6000IC scintillation
6 counter.

MAB34 was more active in the UT-7/EPO cell line: In UT-7/EPO cells MAB34 stimulated cell proliferation with an EC₅₀ of approximately 300 pM (FIG. 2B) as compared
9 to 10 nM for BaF3/EPO-R. The maximum of incorporation was close to the value obtained with EPO. This may be due to the higher concentration of EPO-R molecules on the surface of UT-7/EPO cells, which contain 2400 receptors per cell (Komatsu *et al.* (1993) *Blood* 82,
12 456-464; Nicolis *et al.* (1993) *Exp Hematol* 21, 665-670) compared to 800 for BaF3/EPO-R. The higher the receptor concentration, the lower one should expect the ligand concentration necessary to induce dimerization of the receptors.

- 15 At higher concentrations (above 200 nM) MAB34 antagonizes cell proliferation in both cell lines (FIG. 2). The resulting dose-dependent proliferation curve has a bell-shaped character. MAB34 self-antagonized with IC₅₀ values of approximately 2 µM for both cell
18 lines.

If EPO homodimerizes the receptor, then self-antagonism should be observed at high EPO concentrations. We showed this to be the case using an *in vitro* proliferation assay with
21 BaF3/EPO-R cells. Self-antagonism at high ligand concentrations should also be observed for EPO, if it homodimerizes the receptor. BaF3/EPO-R cells and parental BaF3 cells were assayed over a dose range of 30 pM to 30 µM EPO. Proliferation significantly decreased
24 above 3 µM, but complete inhibition was not observed at the concentrations tested. The estimated IC₅₀ value was approximately 20 µM, representing 74,000 U/ml or 370 µg/ml. This is an extremely high concentration: BaF3/EPO-R cells proliferate with an EC₅₀ of 15 pM,
27 which is six orders of magnitude lower. To confirm that the decrease in signal in the BaF3/EPO-R cells was specific to EPO and not due to toxicity or other artifacts at such high ligand concentrations, parental BaF3 cells were incubated with EPO at identical
30 concentrations and in the presence of IL-3 containing WEHI media. No decrease in the IL-3 dependent proliferation was observed.

EXAMPLE 5: EPO-R AGONIST INDUCES DIFFERENTIATION OF CD34⁺ ERYTHROID PROGENITOR CELLS IN THE PRESENCE OF SCF.

3 MAb34 was tested for its ability to induce differentiation of erythroid progenitor cells. The differentiation of CD34⁺ cells to BFUe is dependent on EPO and SCF (Iscove *et al.* (1974) *J. Cell. Physiol* 83, 309-320). MAb34 was able to induce *in vitro* differentiation of
6 human CD34⁺ erythroid cell precursors.

 To purify CD34⁺ erythroid cell precursors, normal human donors were lymphopheresed according to standard protocols. The lymphopheresed cells were washed,
9 resuspended in Hank's Balanced Salt Solution (HBSS) and separated by density centrifugation over a gradient (ficoll-paque). The low-density cells (LD) were collected from the gradient and washed with HBSS and resuspended in PBS supplemented with 0.5% BSA and 5 mM
12 EDTA at a concentration of 5×10^8 cells/ml. The LD cells were then further purified using a CD34 progenitor Cell Isolation Kit (Qbend/10) made by Miltenyl Biotech GmbH. The *in vitro* BFUe assay was done on purified CD34⁺ cells in methyl cellulose. The medium contains
15 20% FBS, 0.33X IMDM (Gibco), salts, 2-mercaptoethanol, nucleosides, cholesterol, sodium pyruvate, Hu-transferrin, lipids, Hu-insulin, deionized BSA and 100 ng/ml Stem Cell Factor (SCF) (Ponting *et al.* (1994) *Exp. Hematol* 22, 810. To plate out duplicate 1 ml samples, an
18 excess of 3 ml was prepared in sterile polystyrene tubes. Each tube received CD34⁺ cells (10,000 cells/ml), 0.015 ml SCF (20 μ g/ml), and a combination of sample and medium totaling 3 ml. The tubes were mixed and 1 ml was aliquoted onto 35x100 mm tissue culture
21 plates. The plates were incubated at 37°C and 10% CO₂ in a humidified tissue culture incubator. Erythroid colonies (orange to red in color) were scored after 20 days.

 In two independent experiments, duplicate sets of CD34⁺ cells were treated with
24 various concentrations of MAb34, EPO, or a control antibody. After 19 or 20 days, respectively, colonies of BFUe cells were visible in the presence of either MAb34 or EPO, but not in the antibody control (Table 2). The colonies showed typical red color and could
27 be identified as BFUe cells by microscopic analysis. No mixed colonies containing red and white cells were observed. As in the cell proliferation assays described above, EPO was more potent than MAb34. Colonies developed at the lowest EPO concentration tested (1.3 pM),
30 whereas no differentiation was observed below a MAb34 concentration of 7 nM. The absolute number of colonies was higher in the presence of EPO and colonies were larger, containing a higher number of cells. Increasing concentrations of EPO produced larger colonies, whereas
33 the size of colonies formed by MAb34 was not dependent on the concentration of ligand. The

approximate EC_{50} value was 15 nM, and the highest stimulation of differentiation by MAb34 observed was between 22 nM and 220 nM, similar to the maxima observed in the cell proliferation assays (Table 2). In addition, at concentrations above 720 nM MAb34 self-antagonizes in this cell differentiation assay. All these data demonstrate that both cell proliferation and differentiation are driven by ligand induced receptor homodimerization.

TABLE 2
BFUe *In vitro* Differentiation Assay

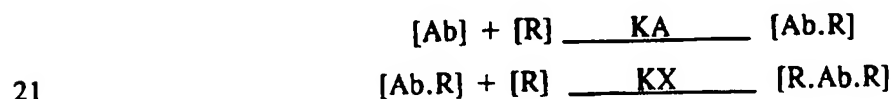
6	Factor	Experiment 1	Colonies	Experiment 2	Colonies
		Conc., [nM]		Conc., [nM]	
9	EPO	0.00254	30 / 22	0.0013	9 / 11
		0.00649	31 / 37	0.0026	31 / 27
		0.0130	49 / 56	0.0066	46 / 62
		0.0259	55 / 61	0.0130	81 / 97
		0.0649	73 / 63	0.0259	142 / 100
12				0.130	133 / 126
15	Mab34			1.8	0/0
		3.6	0 / 0	3.6	0/0
		7.2	0 / 2	7.2	0 / 0
		21.6	12 / 10	14.4	5/6
		36.0	15 / 3	21.6	10 / 7
		72.0	10 / 7	36.0	13/12
		216	11 / 4	72.0	7/15
		360	4 / 3	144	5/14
		720	2 / 6	216	4/13
		1080	0 / 1	360	6 / 9
24					
27					
30	Mab Control			6.9	0 / 0
		34.4	0 / 0	34.4	0 / 0
		206	0 / 0	206	0 / 0
		344	0 / 0	688	0 / 0

Table 2. Purified CD34⁺ cells (10,000 cells per duplicate sample) were incubated in the presence of the indicated sample as described in the methods section. Erythroid colonies were counted after 19 days (experiment 1) and 20 days (experiment 2), respectively.

EXAMPLE 6: AGONIST ACTIVITY OF MAb34 CORRELATES WELL WITH A MODEL FOR ANTIBODY-MEDIATED RECEPTOR DIMERIZATION

Mathematical models have been developed described for the formation of receptor dimers on the cell surface by bivalent ligand antibodies (Perelson, A.S. (1984) in *Cell Surface Dynamics: Concepts and Models* (Perelson, A.S., DeLisi, C., and Wiegel, F.W., eds), pp. 223-276, Marcel Dekker, Inc., New York, NY)) or by GH (Ilondo *et al.* (1994) *Endocrinology* 134, 2937-2403). We investigated how the agonist activity of MAb34 would correlate with the occurrence of receptor dimers predicted by the model of Perelson.

Briefly, Perelson postulates a two step mechanism, where the formation of 1:1 complexes is driven by the affinity constant $K_A (=1/K_D)$. Subsequent dimer formation is dependent on a "cross-linking" constant K_X , which includes K_A but also depends on the effective concentration of receptors on the cell surface and other factors.



The concentration of dimer is given by:

$$[\text{dimer}] = \frac{[\text{Rtotal}]}{2} \left(1 + \frac{1}{2\delta} (1 - \sqrt{1 + 4\delta}) \right)$$

$$\text{where } \delta = \frac{2[\text{AB}][\text{Rtotal}]K_A K_X}{(1 + 2K_A[\text{Ab}])}$$

assuming that the amount of antibody bound is small compared to the total antibody concentration. The concentration of dimer is solely dependent on K_A :

$$[dimer]_{\max} = \frac{1}{2KA} = \frac{1}{2}KA$$

If the percentage of receptor/antibody 2:1 complexes versus the total number of receptors is plotted against the antibody concentration, a symmetrical bell-shaped curve is predicted.

- 3 The maximum of 2:1 complexes occurs at a defined antibody concentration equal to one half of the antibody K_D value.

FIG. 3 fits the data of the MAb34 cell proliferation and differentiation assays to the equation. The resulting bell-shaped curves for the proliferation assays correlate well the assay data. The obtained 2:1 complex maxima were 114 nM for BaF3/EPO-R cells and 26 nM for UT-7/EPO cells. According to the model, this translates to apparent K_D values of 228 nM and 52 nM respectively, in good agreement with the K_D value of 54 nM determined by BIAcore (Pharmacia) analysis (Table 1). A larger number of parallel experiments would minimize data scattering as is apparent to one of ordinary skill in the art. These results demonstrate that the agonist activity of the bivalent monoclonal antibody MAb34 in cell proliferation and differentiation assays is consistent with ligand induced homodimerization of the EPO-R on the cell surface.

15 All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was selectively and individually indicated to be incorporated by reference.

18 The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

- 3 1. An antibody which activates erythropoietin receptors.
2. The antibody of Claim 1 wherein the erythropoietin receptor is mammalian.
- 6 3. The antibody of Claim 2 wherein the erythropoietin receptor is human.
- 9 4. The antibody of Claim 3 which is a monoclonal antibody.
5. The monoclonal antibody of Claim 4 which is murine.
- 12 6. The monoclonal antibody of Claim 4 which is a humanized antibody.
- 15 7. The monoclonal antibody of Claim 4 which is human.
8. The monoclonal antibody of Claim 4 which is an IgG.
- 18 9. The antibody of Claim 4 which has a detectable label.
- 21 10. A bivalent molecule which activates erythropoietin receptors, which bivalent molecule contains at least one binding domain that selectively recognizes an epitope located on the extracellular domain of an erythropoietin receptor, which epitope is
- 24 selectively recognized by the monoclonal antibody produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.
- 27 11. The bivalent molecule of Claim 10 which contains at least two binding domains that selectively recognize the epitope.
- 30 12. The bivalent molecule of Claim 11 wherein the erythropoietin receptor is mammalian.
- 33 13. The bivalent molecule of Claim 12 wherein the erythropoietin receptor is human.

14. The bivalent molecule of Claim 13 which is an antibody.
- 3 15. The antibody of Claim 14 which is a monoclonal antibody.
16. The monoclonal antibody of Claim 15 which is murine.
- 6 17. The monoclonal antibody of Claim 15 which is a humanized antibody.
- 9 18. The monoclonal antibody of Claim 15 which is human.
- 12 19. The monoclonal antibody of Claim 15 which is an IgG.
- 15 20. The antibody of Claim 15 which has a detectable label.
- 18 21. The monoclonal antibody of Claim 15 which is produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.
- 21 22. A hybridoma cell line which produces the monoclonal antibody of Claim 15.
23. The hybridoma cell line of Claim 22 which is Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.
- 24 24. An antibody produced by the hybridoma cell line #34.10.1 deposited at the ATCC as ATCC HB-12088.
- 27 25. A method of treating a condition in a mammal in which diminished erythropoietin receptor activity contributes to the pathology and/or symptomatology of the condition, which method comprises administering to such animal a therapeutically effective amount of an antibody which activates erythropoietin receptors.
- 30 26. The method of Claim 25 in which the antibody is a monoclonal antibody.
- 33 27. The method of Claim 26 in which the condition is anemia.

28. A method of treating a condition in a mammal in which diminished erythropoietin receptor activity contributes to the pathology and/or symptomatology of the condition,
3 which method comprises administering to such animal a therapeutically effective amount
of a bivalent molecule which activates erythropoietin receptors, which oivalent molecule
contains at least one binding domain that selectively recognizes an epitope located on the
6 extracellular domain of an erythropoietin receptor, which epitope is selectively recognized
by the monoclonal antibody produced by Hybridoma #34.10.1 deposited at the ATCC as
ATCC HB-12088.

9

29. The method of Claim 28 in which the bivalent molecule contains at least two
binding domains that selectively recognize the epitope.

12

30. The method of Claim 29 in which the bivalent molecule is an antibody.

15 31. The method of Claim 30 in which the antibody is a monoclonal antibody.

32. The method of Claim 31 in which the monoclonal antibody is that which is
18 produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC HB-12088.

33. The method of Claim 32 in which the condition is anemia.

21

34. A pharmaceutical composition comprising a therapeutically effective amount of the
antibody of Claim 1 in combination with a pharmaceutically acceptable excipient.

24

35. The pharmaceutical composition of Claim 34 in which the antibody is a
monoclonal antibody.

27

36. A pharmaceutical composition comprising a therapeutically effective amount of the
bivalent molecule of Claim 10 in combination with a pharmaceutically acceptable
30 excipient.

37. The pharmaceutical composition of Claim 36 in which the bivalent molecule
33 contains at least two binding domains that selectively recognize the epitope.

38. The pharmaceutical composition of Claim 37 in which the bivalent molecule is an antibody.

3

39. The pharmaceutical composition of Claim 38 in which the antibody is a monoclonal antibody.

6

40. The pharmaceutical composition of Claim 39 in which the monoclonal antibody is that which is produced by Hybridoma #34.10.1 deposited at the ATCC as ATCC

9 HB-12088.

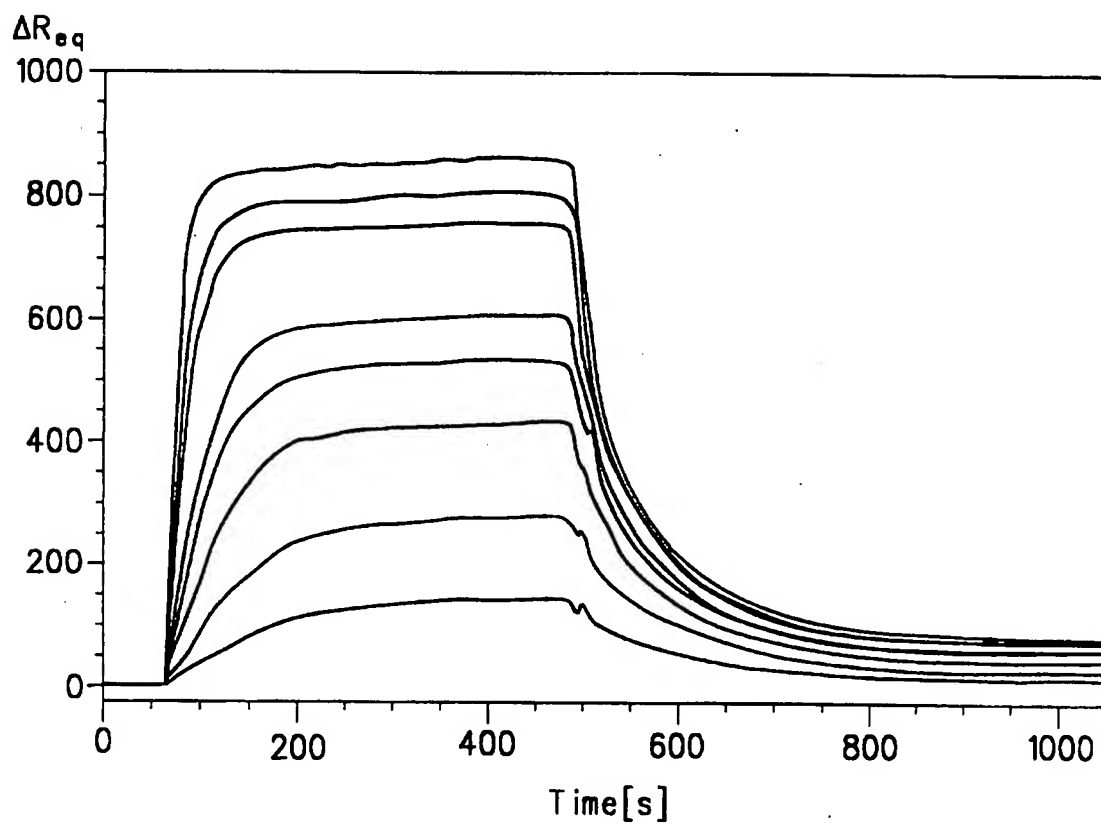


FIG. 1A

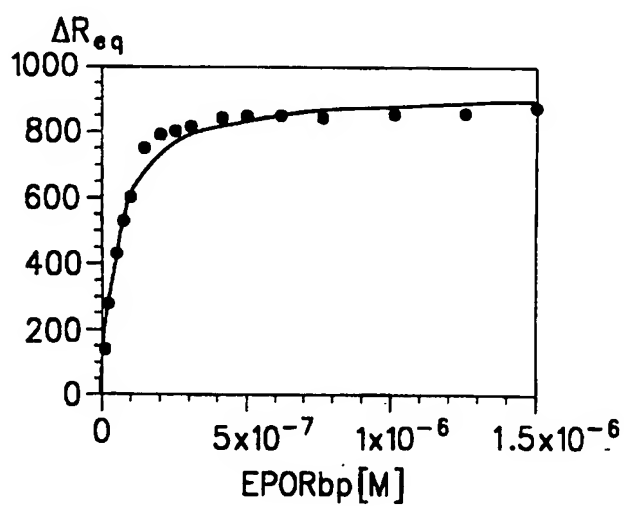


FIG. 1B

2/3

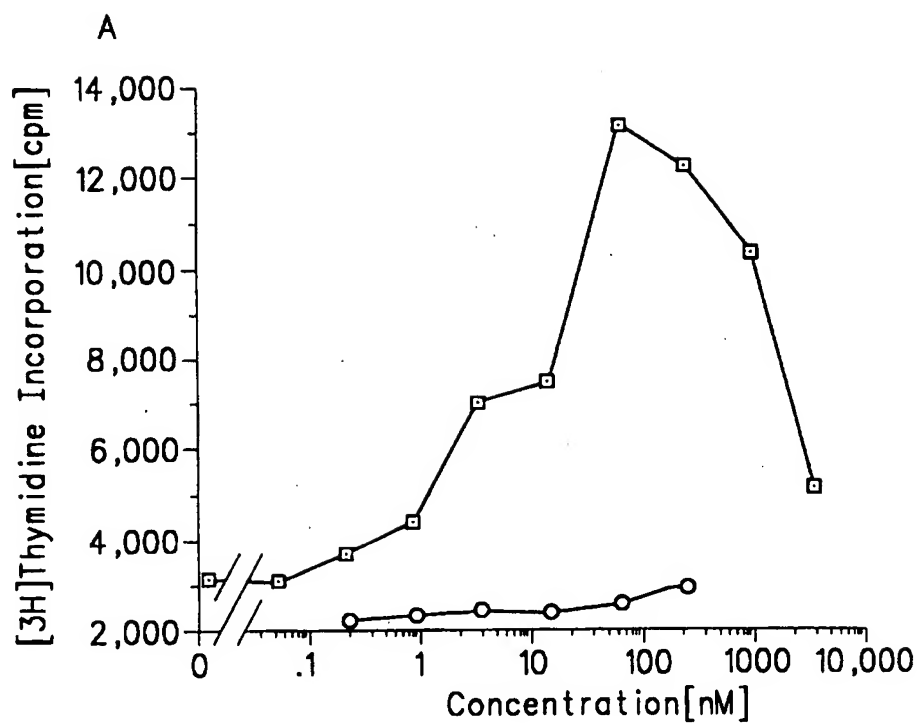


FIG. 2A

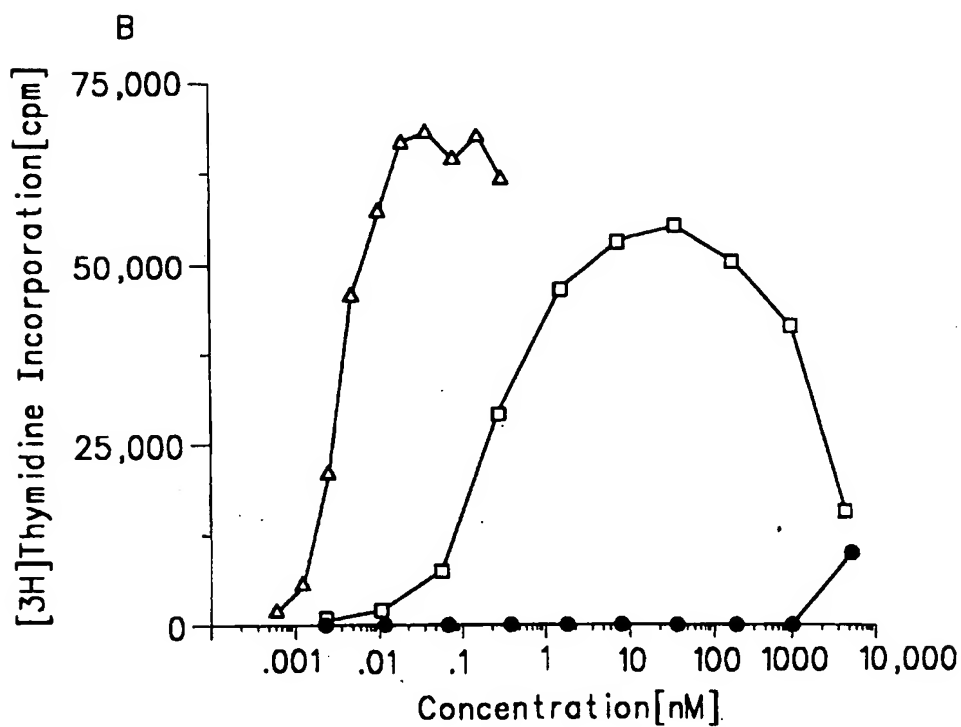


FIG. 2B

3/3

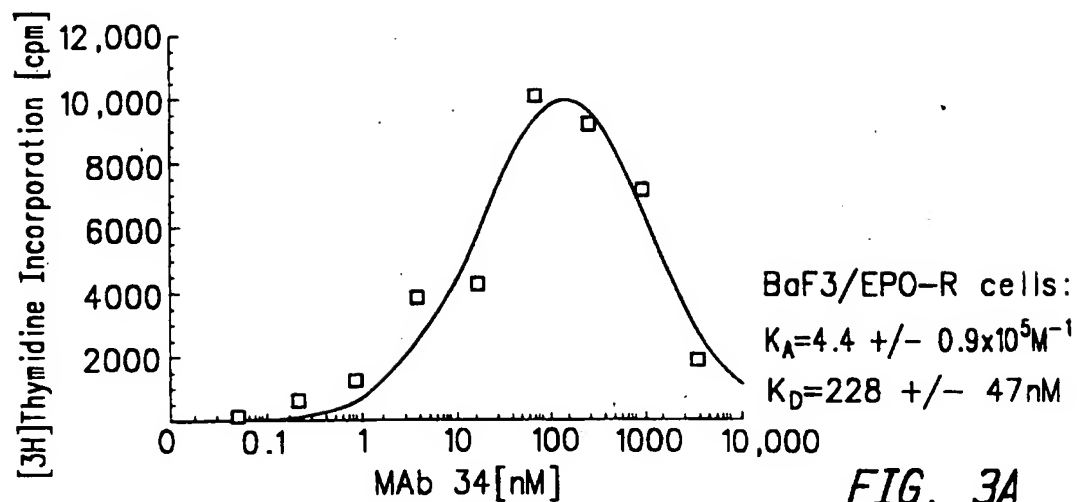


FIG. 3A

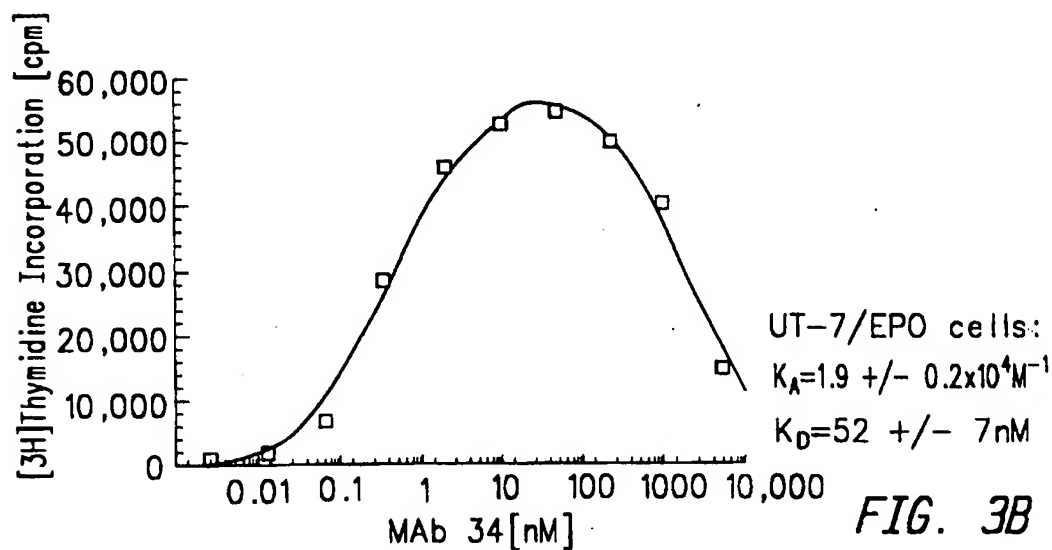


FIG. 3B

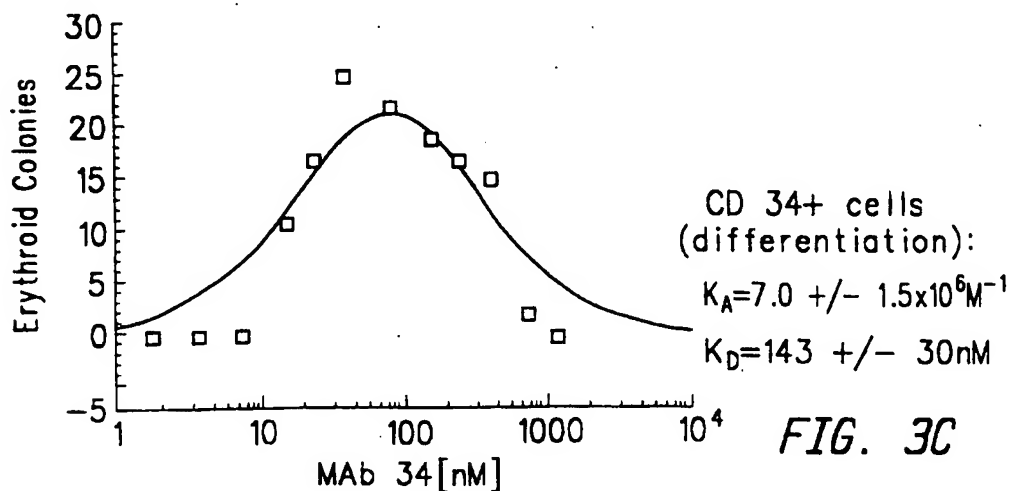


FIG. 3C

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/10564

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/28 C12N5/20 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. ELLIOTT ET AL.: "Isolation and characterization of RHuEPO receptor monoclonal antibodies that activate the EPO receptor." EXPERIMENTAL HEMATOLOGY, vol. 23, no. 8; August 1995, NEW YORK, NY, USA, page 828 XP002044302 see abstract 306 ---	1-24
X	WO 96 03438 A (AMGEN, INC.) 8 February 1996 see the whole document ---	1-40
X	WO 92 21029 A (GENENTECH, INC.) 26 November 1992 see examples 8-10 see claims --- -/--	1-40

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

22 October 1997

Date of mailing of the international search report

18. 11. 97

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/10564

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 90 08822 A (GENETICS INSTITUTE, INC. ET AL.) 9 August 1990 see page 15, line 7 - page 16, line 14 see claims 13-15 ---	1-40
A	H. RUI ET AL.: "JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization." ENDOCRINOLOGY, vol. 135, no. 4, October 1994, PHILADELPHIA, PA, USA, pages 1299-1306, XP002044303 see the whole document ---	1-24
P,X	H. SCHNEIDER ET AL.: "Homodimerization of erythropoietin receptor by a bivalent monoclonal antibody triggers cell proliferation and differentiation of erythroid precursors." BLOOD, vol. 89, no. 2, 15 January 1997, NEW YORK, NY, USA, pages 473-482, XP002044304 see the whole document ---	1-24
P,X	S. ELLIOTT ET AL.: "Activation of the erythropoietin (EPO) receptor by bivalent anti-EPO receptor antibodies." THE JOURNAL BIOLOGICAL CHEMISTRY, vol. 271, no. 40, 4 October 1996, BALTIMORE, MD, USA, pages 24691-24697, XP002044305 see the whole document ---	1-24
P,X	WO 96 40231 A (SMITHKLINE BEECHAM CORP. USA ET AL.) 19 December 1996 see example 10 see claims -----	1-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/10564

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 25-33
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No

PCT/US 97/10564

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9603438 A	08-02-96	AU 3149995 A CA 2195868 A EP 0773962 A	22-02-96 08-02-96 21-05-97
WO 9221029 A	26-11-92	EP 0586549 A JP 6507715 T US 5506107 A	16-03-94 01-09-94 09-04-96
WO 9008822 A	09-08-90	US 5378808 A US 5278065 A	03-01-95 11-01-94
WO 9640231 A	19-12-96	AU 6163196 A	30-12-96